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Effects of reactive oxygen species on cellular wall disassembly of banana fruit during ripening

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Abstract

Fruit softening is generally attributed to cell wall disassembly. Experiments were conducted to investigate effects of various reactive oxygen species (ROS) on *in vitro* cellular wall disassembly of harvested banana fruit. The alcohol-extracted insoluble residue (AEIR) was obtained from the pulp tissues of banana fruit at various ripening stages and then used to examine the disassembly of cellular wall polysaccharides in the presence of superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) or hydroxyl radical ($^{\cdot}OH$) and their scavengers. The presence of $^{\cdot}OH$ accelerated significantly disassembly of cellular wall polysaccharides in terms of the increase in contents of total sugars released and uronic acid, and the decrease in molecular mass of soluble polysaccharides, using gel permeation chromatography. However, the treatment with H_2O_2 or $O_2^{\cdot-}$ showed no significant effect on the disassembly of cellular wall polysaccharides. Furthermore, the degradation of the de-esterified AEIR was more susceptible to $^{\cdot}OH$ attack than the esterified AEIR. In addition, the effect of $^{\cdot}OH$ could be inhibited in the presence of $^{\cdot}OH$ scavenger. This study suggests that disassembly of cellular wall polysaccharides could be initiated by $^{\cdot}OH$ as the solublisation of the polysaccharides increased, which, in turn, accelerated fruit softening.

Keywords: Banana; Cellular wall disassembly; Fruit; Polysaccharides; Reactive oxygen species

1. Introduction

Banana is a typical climacteric fruit. The fruit softens rapidly once ripening is initiated (Jiang, Joyce, & Macnish, 1999; Wills, Klieber, David, & Siridhata, 1990). Fruit softening is generally attributed to cell wall disassembly, particularly due to pectin solubilisation (Brummell & Harpster, 2001; Huber, 1983; Lohani, Trivedi, & Nath, 2004). A previous study suggested that some hydrolysis-related enzymes were the major factors initiating cellular wall disassembly *in vivo* of harvested fruits (Abu-Goukh & Bashir, 2003). Involvement of polygalacturonase (PG) or/and pectin methyl esterase (PME) in enzymatic disassembly of cellular wall has been widely reported (Nikolic & Mojovic, 2007; Verlent, Smout, Duvetter, Hendrickx, & Loey, 2005). In addition, pectate lyase (PL) (Payasi, Misra, & Sanwal, 2006), cellulase (Abu-Goukh & Bashir, 2003), β-galactosidase (Lazan, Ng, Goh, & Ali, 2004), transglycosylases (Rose, Braam, Fry, & Nishitani, 2002) or expansin proteins (Wang, Lu, Jiang, Luo, Jiang, & Joyce, 2006) may play a role in cell wall disassembly during fruit softening.

Recent research showed that the disassembly of cellular wall might be related to non-enzymatic scission of polysaccharides (Dumville & Fry, 2003). ROS, including $O_2^{\cdot-}$,

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H_2O_2 and ·OH accumulates during fruit ripening (Vicente, Martinez, Chaves, & Civello, 2006). Production of ·OH by H_2O_2 in the presence of Fe^{2+} ions (Fenton's reagent) has shown to degrade cellular wall substances (Halliwell, 1965). Breakdown of cellular wall polysaccharides because of ·OH had been found by *in vitro* tests (Fry, 1998; Schweikert, Liszkay, & Schopfer, 2000). Schopfer (2001) reported that ·OH could cause cellular wall loosening and resulted in elongation of living coleoptile or hypocotyls. Unfortunately, very little information about the effects of ROS on disassembly of cellular wall of harvested fruit during ripening is available. Based on the formation of ROS during fruit ripening, it is hypothesized that depolymerization and solubilisation of cellular wall polysaccharides can be initiated by ROS, which, in turn, can accelerate fruit softening. Accordingly, the effects of ROS on cellular wall disassembly of pulp tissues of banana fruit in relation to various ripening stages were investigated. This work could be helpful for understanding the biochemical and physiological mechanism of fruit softening during storage.

2. Materials and methods

2.1. Plant material

Hands of mature green banana fruit (*Musa* spp., AAA group, cultivar 'Brazil') were obtained from a local farm in Guangzhou, China. Fruit were selected for freedom from visual defects and for uniformity of weight and shape at the similar maturity. Fruits were cut into fingers and then dipped in a 0.1% Sportak® (prochloraz, Bayer) fungicide solution for 3 min to control the postharvest diseases. After air-drying, these fruits were kept at 25 ± 1 °C and 90% relative humidity. At 0, 10, 15, 17 and 20 days when fruit ripening reached stage I (mature green), II (green), III (green > yellow), IV (yellow > green) and V (yellow), respectively, ten fingers of these fruits were sampled and then peeled. The entire pulp tissues were cut into small pieces, frozen immediately in liquid nitrogen and kept at −80 °C.

2.2. Preparation of AEIR

AEIR was prepared by the method of Vierhuis, Schols, Beldman, and Voragen (2000). The frozen pulp tissues (100 g) were blended with 300 ml of 95% (v/v) ethanol using a homogenizer and then incubated in boiling water for 15 min to inactivate endogenous enzymes. After cooling rapidly in an ice bath, the mixture was centrifuged at 4000*g* for 15 min. The residue was washed sequentially with 200 ml of a mixture of chloroform/methanol (1:1, v/v) and 200 ml of acetone. The insoluble residue was re-extracted twice with 70% ethanol, filtered and dried at 40 °C. The dried powder was considered as the AEIR fraction and then stored in desiccators. In this study, banana starch could be removed well from the AEIR preparation by the method of Vierhuis et al. (2000) and no starch was detected using the KI–I_2 method.

2.3. Effects of ROS and their scavengers on contents of the total sugars released and uronic acid from AEIR

The AEIR from banana fruit pulp at five ripening stages were washed with 100 mM acetate buffer (pH 5.0) overnight at 25 °C by stirring to remove soluble polysaccharides and the mixture solution was filtered. The insoluble residue was collected and then re-suspended in 40 mM acetate buffer (pH 5.0) to obtain an AEIR suspension (1%, w/v). Effects of ROS and their scavengers on contents of the total sugars released and uronic acid from various AEIR fractions were investigated. Each reaction mixture contained 0.2 ml of 1% AEIR suspension and 0.7 ml of 40 mM sodium acetate buffer (pH 5.0) and the reaction mixture was then incubated with 0.1 ml of ROS or their scavenger solutions. In this study, the final concentration of ROS was as 1 mM paraquat ($O_2^{\cdot-}$ production) (Takizawa, Komori, Tampo, & Yonaha, 2007), 1 mM H_2O_2 or 1 mM $FeSO_4$ and 1 mM H_2O_2 (·OH production) (Halliwell, 1965), while the final concentration of their scavengers was 10 mM glutathione (GSH, reduced) for $O_2^{\cdot-}$ (Horstman, Wrona, & Dryhurst, 2002), 10 mM dimethyl sulphoxide (DMSO) for ·OH (Srivastava & Chan, 2007; Herdener, Heigold, Saran, & Bauer, 2000) or 10 units of catalase (CAT) for H_2O_2 (Srivastava & Chan, 2007). Water instead of ROS or their scavenger solutions was the control. The mixtures were shaken for 12 h at 120 rpm and 25 °C, and the insoluble cellular wall materials were removed by filtration. The filtrate was used for measurements of total sugar and uronic acid contents.

2.4. Effects of Fe^{2+}/H_2O_2 concentration and pH value on contents of total sugars released and uronic acid from AEIR

The effects of Fe^{2+}/H_2O_2 concentrations and pH values on the contents of total sugars released and uronic acid from AEIR of banana fruit at mature green stage were examined. 0.7 ml of 40 mM sodium acetate buffer at various pH values of 3.7, 5.0, 5.2 and 5.6 or 40 mM sodium phosphate buffer at pH 7.0 and 0.2 ml of 1% AEIR suspension were incubated with ·OH reaction system ($FeSO_4/H_2O_2$). Various concentrations of Fe^{2+}/H_2O_2 at 0 (control), 0.1, 0.5, 1 and 5 mM were used, respectively. The reaction and measurement of contents of total sugars released and uronic acid were the same as the above-mentioned procedure.

2.5. Effect of DMSO concentration on contents of ·OH-induced total sugars released and uronic acid from AEIR of mature green banana fruit

Each reaction mixture contained a volume of 1 ml. 0.2 ml of 1% AEIR suspension, 1 mM Fe^{2+}/H_2O_2 and 40 mM sodium acetate buffer (pH 5.0) were incubated with DMSO at 0 (control), 0.1, 1, 10 and 50 mM, respectively. The reaction and measurement of contents of total sugars released and uronic acid were the same as the above-mentioned procedure.

2.6. Alkaline hydrolysis

Alkaline hydrolysis was conducted by the method of Dumville and Fry (2003). The AEIR suspension from mature green fruit was incubated for 5 h with 0.1 M NaOH. Then, the pH value of the mixture solution was adjusted to 5.0 with acetic acid. Another AEIR suspension was performed with the same amount of acetic acid and NaOH but in a reverse order to ensure no occurrence of alkaline hydrolysis of the AEIR suspension.

2.7. Gel permeation chromatography

The alkaline-hydrolysed and non-hydrolysed AEIR samples were incubated with or without ·OH (produced by Fenton reaction: 1 mM $FeSO_4$ and 1 mM H_2O_2) and then fractionated by gel permeation chromatography. Gel permeation chromatography was performed by the method of Manrique and Lajolo (2004) with minor modification. The incubated AEIR solutions were respectively loaded onto a 1 × 80 cm column packed with Sepharose 4B resin (dextrans fractionation range: 10–5000 kDa, Pharmacia, Sweden), and then eluted with 40 mM sodium acetate buffer (pH 5.0) containing 0.1% NaCl and 0.02% NaN_3 at a flow rate of 12 ml/h. Fractions were collected every 10 min. The molecular mass calibration curve was obtained using standard dextrans with mean molecular weights of 500, 70, 40 and 10 kDa (Pharmacia, Sweden).

2.8. Measurement of uronic acid

Uronic acid contents were measured by the methods of McCready and McComb (1952) and McComb and McCready (1952), with slight modifications. Sample (500 μl) was ice-cooled and then added to 2.5 ml of 98% (v/v) H_2SO_4. The mixture solution was incubated in boiling water for 10 min. After cooling to room temperature (25 °C), 250 μl of 0.15% (w/v) carbazole was added to the mixture solution. The absorbance at 530 nm was recorded after 10 min of incubation and the content of uronic acid was then calculated using galacturonic acid as the standard.

2.9. Measurement of total sugar content

Contents of total sugars were determined by the method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956). Briefly, sample (500 μl) was incubated for 30 min with 500 μl of 5% (w/v) phenol and 2.5 ml of 98% (v/v) H_2SO_4. The absorbance at 490 nm was recorded and then the content of total sugars was calculated using glucose as the standard.

2.10. Data handling

The experiments were arranged in a completely randomized design, and each comprised of three replicates. The data were expressed as means ± standard error (SE) and analysed by SPSS (Version 13). One-way analysis of variance (ANOVA) was carried out to test any significant differences between the means. Least significant differences (LSDs) were calculated to compare significant treatment effects at 5% level.

3. Results and Discussion

3.1. Effects of ROS and their scavengers on cell wall disassembly of banana fruit at various ripening stages

Effects of ROS and their scavengers on contents of total sugars released and uronic acid from the AEIR suspensions were summarized in Tables 1 and 2, respectively. The presence of ·OH markedly increased the contents of total sugars released and uronic acid from AEIR at the early stage of fruit ripening, which indicated an enhanced-solubilisation of cell wall polysaccharides. Similar results on the depolymerization of cellular wall polysaccharides by ·OH

Table 1
Effects of ROS and their scavengers on contents of total sugars released from alcohol-extracted insoluble residue (AEIR) of pulp tissues of banana fruit at various ripening stages

	Concentration (mmol/l)	Total sugars released (μg/mg AEIR)				
		I	II	III	IV	V
Control	0	34.78 bc	71.65 c	88.55 bc	119.96 b	195.00 d
Paraquat	1	33.42 c	79.23 bc	88.83 b	120.67 b	208.42 c
H_2O_2	1	44.49 bc	83.24 b	85.88 bc	103.44 d	194.40 d
·OH[A]	1	81.90 a	119.91 a	130.33 a	159.45 a	245.12 a
CAT[B]	[C]	36.69 bc	76.34 bc	81.36 c	112.49 c	209.56 c
DMSO[D]	10	38.22 bc	74.05 c	88.17 bc	110.86 c	195.49 d
GSH[E]	10	35.27 bc	77.92 bc	88.39 bc	115.22 bc	219.32 b

The means within a column followed by the same letter were not significantly different at 5 % level.
[A] ·OH was produced by Fenton reaction of 1 mM Fe^{2+} with 1 mM H_2O_2.
[B] CAT (Catalase) was used as a enzyme of scavenging H_2O_2.
[C] Indicated a 10-unit CAT activity.
[D] DMSO (Dimethyl sulphoxide) was used as a scavenger of ·OH.
[E] GSH (Glutathione) was used as a scavenger of $O_2^{\cdot-}$. I, II III, IV and V were the five ripening stages of banana fruit during storage, corresponding to mature green, green, green > yellow, yellow > green and yellow in skin color, respectively.

Table 2
Effects of ROS and their scavengers on contents of uronic acid from alcohol-extracted insoluble residue (AEIR) of pulp tissues of banana fruit at various ripening stages

	Concentration (mmol/l)	Uronic acid (μg/mg AEIR)				
		I	II	III	IV	V
Control	0	22.41 b	40.13 b	38.79 bc	71.05 b	81.93 a
Paraquat	1	18.14 b	37.02 b	32.37 c	67.87 b	106.00 a
H_2O_2	1	14.35 b	39.83 b	33.66 c	87.31 ab	102.76 a
OH[A]	1	46.67 a	63.41 a	66.90 a	94.52 a	103.25 a
CAT[B]	[C]	21.80 b	41.23 b	33.29 c	76.12 ab	99.77 a
DMSO[D]	10	18.08 b	43.62 b	37.75 bc	73.19 b	106.12 a
GSH[E]	10	17.34 b	43.49 b	46.12 b	67.02 b	107.29 a

The means within a column followed by the same letter were not significantly different at 5% level.
[A] ·OH was produced by Fenton reaction of 1 mM Fe^{2+} with 1 mM H_2O_2.
[B] CAT (Catalase) was used as a enzyme of scavenging H_2O_2.
[C] Indicated a 10-unit CAT activity.
[D] DMSO (Dimethyl sulphoxide) was used as a scavenger of ·OH.
[E] GSH (Glutathione) was used as a scavenger of $O_2^{\cdot-}$. I, II III, IV and V were the five ripening stages of banana fruit during storage, corresponding to mature green, green, green > yellow, yellow > green and yellow in skin colour, respectively.

produced by Fenton reaction (Fry, 1998; Fry, Dumville, & Miller, 2001; Fry, Miller, & Dumville, 2002) and peroxidase (Schweikert, Liszkay, & Schopfer, 2002) were observed. The increase in contents of total sugars and uronic acid could indicate enhanced-solubilisation of cellular wall polysaccharides, such as pectin and xyloglucan (Lashbrook, 2005). However, no significant difference (LSD, $P = 0.05$) in contents of the total sugars released and uronic acid between control and treatment with paraquat or H_2O_2 was observed. This study suggested that $O_2^{\cdot-}$ and H_2O_2 had no significant effect on cellular wall disassembly of banana fruit *in vitro* and corroborated the report of Sonntag (1987), who found that $O_2^{\cdot-}$, H_2O_2 and singlet oxygen did not influence polysaccharide degradation.

In this investigation, the effect of ROS scavengers on contents of total sugars released and uronic acid from the AEIR suspension was further examined. GSH, CAT and DMSO were used as the scavengers of $O_2^{\cdot-}$, H_2O_2 and ·OH, respectively. The presence of 10 mM GSH, 10 mM DMSO or 10-unit CAT had no significant effect on the contents of total sugars released and uronic acid from the AEIR suspension of banana fruit at various ripening stages.

3.2. Effects of various Fe^{2+}/H_2O_2 concentrations and pH values on cellular wall disassembly of mature green banana fruit

The AEIR from mature green fruit was used to further investigate the effects of various Fe^{2+}/H_2O_2 concentrations and pH values on cellular wall disassembly. The contents of total sugars released and uronic acid increased significantly (Tables 3 and 4) with increasing Fe^{2+}/H_2O_2 concentrations, which indicated a dose-dependent enhanced effect of ·OH on cellular wall disassembly. Furthermore, the contents of the total sugars released were the most manifest at pH 5.2. The results confirmed the previous study of Fry (1998), who suggested that ·OH formation in the apoplastic space of plant tissues could act as a site-specific oxidant which played a role in cellular wall loosening processes. Schweikert et al. (2000) reported that ·OH production effectively cleaved polysaccharides *in vitro* at physiological pH.

Table 3
Effects of various Fe^{2+}/H_2O_2 concentrations and pH values on contents of total sugars released from alcohol-extracted insoluble residue (AEIR) of pulp tissues of mature green banana fruit

Concentration (mmol/l)	Total sugars released (μg/mg AEIR)				
	pH 3.7	pH 5.0	pH 5.2	pH 5.6	pH 7.0
Control	43.00 d	34.46 e	38.71 d	40.73 d	33.19 e
0.1	46.24 d	44.16 d	44.11 d	41.16 d	46.13 d
0.5	72.52 c	74.38 c	71.05 c	76.39 c	59.54 c
1	87.68 b	97.33 b	95.97 b	93.63 b	70.56 b
5	161.93 a	215.1 a	219.84 a	212.07 a	196.04 a

The means within a column followed by the same letter were not significantly different at 5% level.

Table 4
Effects of various Fe^{2+}/H_2O_2 concentrations and pH values on the contents of uronic acid from alcohol-extracted insoluble residue (AEIR) of pulp tissues of mature green banana fruit

Concentration (mmol/l)	Uronic acid (μg/mg AEIR)				
	pH 3.7	pH 5.0	pH 5.2	pH 5.6	pH 7.0
Control	22.39 d	20.83 d	26.94 d	15.42 d	21.61 c
0.1	31.33 cd	29.68 cd	28.40 d	26.45 d	37.38 b
0.5	40.01 bc	44.84 bc	42.82 c	49.48 c	39.28 b
1	47.89 b	51.87 b	62.19 b	57.79 b	49.67 b
5	65.61 a	141.2 a	106.37 a	109.67 a	106.98 a

The values within a column followed by the same letter were not significantly different at 5% level.

3.3. Effect of DMSO concentrations on contents of ·OH-induced total sugars released and uronic acid from AEIR of mature green banana fruit

DMSO at different concentrations was used as a scavenger of ·OH to further validate the effects of ·OH on the

disassembly of cellular wall polysaccharides in terms of total sugars released (Dubois et al., 1956) and uronic acid (McCready & McComb, 1952; McComb & McCready, 1952). DMSO significantly decreased the contents of total sugars released and uronic acid from the AEIR suspension of mature green fruit when the concentration used increased (Table 5), which indicated that ·OH scavenger at high concentration inhibited the disassembly of cellular wall polysaccharides of banana fruit pulp. A similar result was obtained in harvested tomato fruit by Dumville and Fry (2003).

Table 5
Effect of various DMSO (Dimethyl sulphoxide, a scavenger of ·OH) concentrations on contents of ·OH-induced total sugars released and uronic acid from alcohol-extracted insoluble residue (AEIR) of pulp tissues of banana fruit at mature green stage

Concentration (mM)	Total sugars released (μg/mg AEIR)	Uronic acid (μg /mg AEIR)
0	86.26 a	42.39 a
0.1	84.46 a	38.73 a
1	85.61 a	32.31 ab
10	78.03 ab	23.79 bc
50	73.67 b	20.61 c

The means within a column followed by the same letter were not significantly different at 5% level.

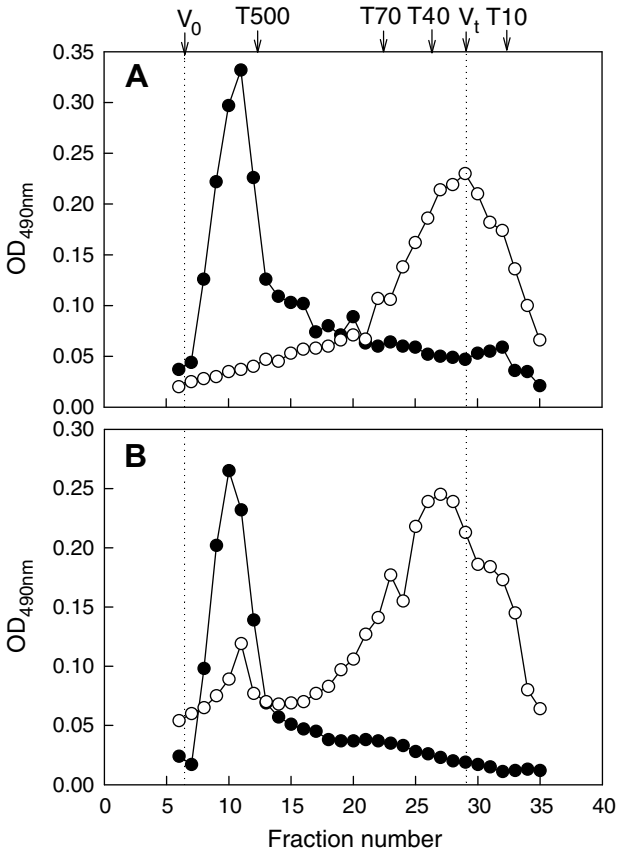

Fig. 1. Effect of ·OH production on the molecular mass (Mr) of the alkaline-hydrolysed alcohol-extracted insoluble residue (A) and non-hydrolysed alcohol-extracted insoluble residue (B) of pulp tissues of mature green banana fruit. V_0 and V_t were the empty volume and total volume of column while Mr of standards T500, T70, T40 and T10 were 500, 70, 40 and 10 kDa, respectively. (○) treatment with ·OH and, (●) treatment without ·OH.

3.4. Effect of ·OH on the molecular mass (Mr) of non-hydrolysed AEIR and alkaline-hydrolysed AEIR of mature green banana fruit

The soluble-polysaccharide molecular mass of the alkaline-hydrolysed AEIR (Fig. 1A) and non-hydrolysed AEIR (Fig. 1B) after incubation with and without 1 mM $FeSO_4$/H_2O_2 were determined by gel permeation chromatography. The elution peaks of the alkaline-hydrolysed AEIR suspensions after incubation with and without ·OH were 21–22 ml (Mr = about 708 kDa) and 57–58 ml (Mr = about 21 kDa), respectively. Similarly, the elution peaks of the non-hydrolysed AEIR suspensions after incubation with and without ·OH were 19–20 ml (Mr = about 859 kDa) and 53–54 ml (Mr = about 32 kDa), respectively. It was therefore suggested that ·OH increased the polysaccharide solubilisation of the alkaline-hydrolysed AEIR and non-hydrolysed AEIR. Wakabayashi, Chun, & Huber (2000) reported that alkaline hydrolysis of the AEIR suspension occurred easily in ester bonds and resulted in de-esterification of polysaccharides (pectins). In this study, the de-esterified AEIR was more susceptible to ·OH attack than the esterified AEIR (Fig. 1), which was in agreement with the report of Dumville and Fry (2003) who found that the enhanced de-esterified pectin increased the susceptibility of tomato fruit softening to ascorbate-induced (·OH-mediated) attack.

4. Conclusions

An enhanced effect of ·OH on the depolymerization of cellular wall polysaccharides of AEIR from pulp tissues of banana fruit was obtained at about pH 5.0, in terms of the contents of total sugars released and uronic acid, and the molecular mass of the soluble polysaccharides. Moreover, the de-esterified AEIR was more susceptible to ·OH attack. DMSO (a scavenger of ·OH) at high concentration partially inhibited the degradation of cellular wall polysaccharides of AEIR from mature green banana fruit. However, the presence of H_2O_2 or $O_2^{\cdot-}$ exhibited no significant effect on the degradation of cellular wall polysaccharides of AEIR. Further work is needed to better understand fruit softening concerning the depolymerization of cellular wall polysaccharide by ·OH attack, particularly in polysaccharide structure change.

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